Measurement of telomere length by the Southern blot analysis of terminal restriction fragment lengths

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In this protocol we describe a method to obtain telomere length parameters using Southern blots of terminal restriction fragments (TRFs). We use this approach primarily for epidemiological studies that examine leukocyte telomere length. However, the method can be adapted for telomere length measurements in other cells whose telomere lengths are within its detection boundaries. After extraction, DNA is inspected for integrity, digested, resolved by gel electrophoresis, transferred to a membrane, hybridized with labeled probes and exposed to X-ray film using chemiluminescence. Although precise and highly accurate, the method requires a considerable amount of DNA (3 µg per sample) and it measures both the canonical and noncanonical components of telomeres. The method also provides parameters of telomere length distribution in each DNA sample, which are useful in answering questions beyond those focusing on the mean length of telomeres in a given sample. A skilled technician can measure TRF length in ~130 samples per week.

INTRODUCTION

A body of work points to the involvement of human telomeres in a spectrum of diseases, ranging from rare monogenic diseases to common complex traits such as atherosclerosis and other age-related maladies[1–3]. The considerable scientific and medical interest in telomere biology has culminated recently in the awarding of the 2009 Nobel Prize in Physiology or Medicine to Elizabeth Blackburn, Carol Greider and Jack Szostak in recognition of their work in demonstrating the fundamental importance of telomere maintenance to human health and aging[4]. The use of reliable methods to measure telomere length in diverse settings is essential for the advancement of the telomere biology field.

A quantitative method to measure mean telomere length using the length distribution of the terminal restriction fragments (TRFs) obtained by Southern blots was reported 20 years ago[5]. Other methods of telomere length measurement have been developed since then, including quantitative fluorescence *in situ* hybridization (Q-FISH), which can quantify telomere length signals not only of cells in interphase but also of individual metaphase chromosomes[6,7], Flow-FISH, which uses flow cytometry to measure telomeres in individual cells[8], quantitative PCR (qPCR) of mean telomere length[9,10], and PCR of single telomere length analysis (STELA)[11,12]. We have adopted the Southern blot method for the measurement of TRFs in our epidemiological and clinical research[13–17] as well as in our search for genes that explain interindividual variation in telomere length in the general population[18,19].

In this protocol we focus on how to perform the TRF length analysis, underscoring its advantages and disadvantages in epidemiological research. A flow chart summarizing the procedures and time required for Southern blot analysis of TRF length is shown in **Figure 1**. As most epidemiological studies have measured telomere length in leukocytes or their subsets, the description below focuses on the measurement of leukocyte telomere length (LTL); however, the method can be applied to other cells and tissues from humans (or other animals with known telomere sequence), provided that sufficient DNA per sample is available, the lengths of the TRFs are within the discernible range of the method and the genome does not contain large amounts of heterogeneous telomere-like sequences within the bulk DNA.

With the exception of STELA and the Southern TRF method, other techniques mentioned above estimate length indirectly from a signal that arises from nonresolved telomeres of unknown individual sizes. Moreover, variables influencing the signal from telomeres and reference standards are not well understood compared with those from the Southern TRF method. These limitations are acceptable to researchers who have the capacity to process and analyze samples on the same occasion in order to minimize interassay variability, and who are primarily interested in rank order of telomere length or in relative telomere length changes rather than in accurate, absolute telomere length. However, whether assays are run on a small or a large scale, both bench and clinical researchers need to be concerned with the practical features of a given method (e.g., cost, throughput, requirement for highly skilled labor) as well as the full range of its assay characteristics, including precision, accuracy, specificity, limit of detection, linearity and dynamic range. Accuracy in measuring the absolute length of telomeres is especially important for facilitating comparisons of findings across studies in diverse populations and in setting thresholds for telomere size that might eventually impact clinical decisions (if and when parameters of telomere length are introduced into medical practice).

In that light, it is useful to consider the practicality of the various methods of measuring telomere length in epidemiological settings. Although highly useful in addressing specific questions that relate to telomere biology, the ultimate use of Q-FISH in determining telomere length and STELA in epidemiology is uncertain at

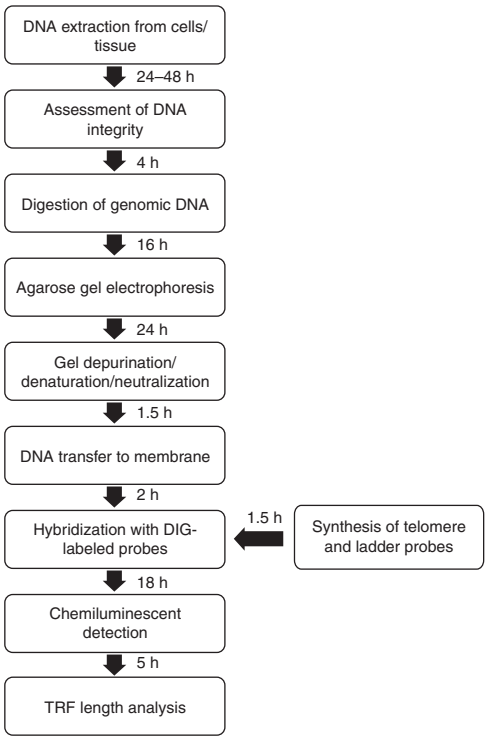

Figure 1 | Summary of procedures involved in TRF length analysis, along with approximate time needed.

present. That is because reported data do not provide estimates of the impact of confounding factors, such as tissue collection, storage and interassay variation on results generated by these methods in large cohort studies. The Flow-FISH approach has been successfully used in epidemiological settings to measure telomere length in leukocytes[20,21]. However, Flow-FISH requires intact cells (nuclei). Moreover, granulocytes are unstable in *ex vivo* preparations and also have a short biological life (hours) *in vivo*. Therefore, samples must be processed as promptly as possible if telomere length in granulocytes is of interest. These features limit the use of the Flow-FISH method in epidemiological research (**Table 1**).

These constraints explain the use of Southern blot analysis of the TRFs or qPCR in most epidemiological studies (**Table 1**). Although all telomere length measurement methods have shortcomings, the debate about the use of qPCR versus Southern blot analysis in epidemiology has been particularly intense. The qPCR method provides the ratio of the telomeric product (T), normalized for a single-copy gene (S) that is amplified in concert with the telomeres. Thus, the T/S value provides information, in relative terms, on telomere length. On one level, using qPCR to measure telomere length makes sense. The method is relatively cheap, has high throughput and requires little DNA. In contrast, the Southern blot analysis of TRFs requires a considerable quantity of DNA and is cumbersome, labor intensive and costly. Consequently, since the introduction of the qPCR method 8 years ago[9], its use has rapidly spread among telomere epidemiologists. However, questions have been raised regarding its reliability, particularly in the context of interassay coefficients of variation (CVs), as compared with Southern blots. In this article, we steer clear of the debate, but we emphasize that an impartial comparison of the use of the qPCR and Southern blot methods for measuring telomere length in epidemiological research is long overdue.

To put the issue of interassay CVs in perspective, what can one expect in epidemiological research from an LTL measurement with an interassay CV of 2% or more? A typical mean LTL in subjects of age 55 years might be 6.0 kb with an s.d. of 0.6 kb. If LTL was measured without error and one wanted to detect a 0.20-kb difference between two groups of subjects (equivalent to a 10-year age difference, as LTL shortening per year is ~0.02 kb, although there is a wide range of variation in the rate of shortening), one would need 141 subjects in each group for 80% power and an α of 5%. If the CV were 2% instead of 0%, the overall pooled s.d. would be 0.612. With this s.d., one could detect a mean difference between groups of 0.204 kb. For a 5% CV, the detectable mean difference would only be 0.223 kb, which increases to 0.283 kb for a 10% CV. As some of the methods used to measure LTL have a 10% CV, the detectable difference between groups, given a constant sample size, increases by 0.083 kb. This is equivalent to about 4 additional years of LTL shortening; it greatly reduces the ability to detect significant differences between groups with respect to variables that affect LTL less than LTL shortening in the course of 10 years. It would take larger sample sizes (e.g., $n = 282$ in each group for a 10% CV) to overcome the loss of power due to a higher CV. Indeed, most variables show much less of an effect on LTL than a 10-year age difference.

Experimental design

Sample type and preparation. A minimum of 3 µg of DNA is required for accurate measurement of telomere length using this method.

TABLE 1 | Advantages and disadvantages of three methods used to measure telomeres in epidemiological settings.

	Southern blot	qPCR	Flow-FISH
Advantages	Can measure telomere length distribution Coefficient of variation <2% Measurements expressed in absolute values (kb)	Low cost High throughput Little DNA required (50 ng per sample)	Can measure average telomere length in each cell Telomere length can be determined in specific cell populations
Disadvantages	Labor intensive Costly Greater quantity of DNA required (3 µg per sample) Presence of subtelomeric DNA in TRFs confounds absolute telomeric length estimate	Only average telomere length is measured Coefficient of variation >2% Lack of good reference standards makes absolute telomere length measurement difficult	More complex clinical sample processing Requires highly skilled, intensive labor Costly Measurements expressed in relative values (fluorescence intensity)

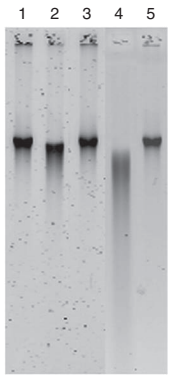

Figure 2 | Evaluation of DNA integrity. DNA samples (10 ng) were resolved on a 1% (wt/vol) agarose gel at 200 V for 60 min. Lanes 1, 3 and 5 contain DNA that is intact, appearing as single compact crowns that have migrated in parallel. Lanes 2 and 4 contain degraded DNA. In lane 2, the crown is shifted forward, whereas in lane 4 the crown is fuzzy with a tail of DNA smear; DNA samples in both lanes are unsuitable for TRF length analysis.

DNA extraction. Multiple methods are available for the isolation of genomic DNA from cells and tissues of eukaryotes. All methods share three basic steps: lysis of cells or tissue, removal of protein, RNA and other contaminants, and recovery of DNA. Detailed in this protocol are the two methods used by most laboratories to prepare DNA for TRF length analysis: phenol-chloroform organic extraction and a commercially available 'salting-out' system. Phenol-chloroform extraction provides high-quality, high-yield DNA, making this technique especially suitable for tissue samples in which there is a limited amount of starting material[22]. However, this method has the substantial disadvantage of reagent toxicity: phenol and chloroform must be handled with caution. In addition, this technique is time consuming and is not easily adapted for processing multiple samples, making it cumbersome for use in epidemiological studies. Therefore, we prefer to use a commercially available DNA extraction kit in our laboratory whenever possible. Contaminants and enzyme inhibitors are removed by salt precipitation, and the resulting purified DNA is available for immediate use. This kit is scalable and can easily be adapted for high-throughput processing of multiple samples, thus making it especially suitable in epidemiological settings.

Evaluation of DNA integrity. A critical step preceding sample digestion and TRF length analysis is verification of DNA integrity. Skipping this step undermines the validity of the results, as very often we receive inadequate DNA samples due to a host of problems related to sample collection, storage and perhaps shipment. Samples are run side by side using agarose gel electrophoresis, and DNA is visualized with a nucleic acid stain. DNA must appear as a single compact crown-shaped band that migrates in parallel with the other samples on the gel. A sample that appears as a smear or has a forward shift in its crown in comparison with other samples is probably degraded and thus unsuitable for TRF length analysis (**Fig. 2**). The importance of checking DNA integrity cannot be overemphasized, as performing TRF length analysis on degraded DNA will yield inaccurately short telomeric measurements. This step is particularly vital for epidemiological studies; it is strongly recommended that the integrity of a random group of DNA samples from within each cohort be evaluated before undertaking large-scale measurements of telomeres in the entire group. Once the study is undertaken, all samples are subjected to the DNA integrity test.

Digestion of genomic DNA. Mammalian telomeres are a tandem array of TTAGGG repeats at the ends of chromosomes. Adjacent to this area is a subtelomeric region comprising noncanonical repeats. The principle of TRF length analysis lies in the relative lack of restriction sites in these regions. By digesting genomic DNA with restriction endonucleases that cut frequently within chromosomal loci but not within telomeric repeats, DNA is enriched for high–molecular-weight telomeric fractions, with the remainder reduced to small fragments of < 800 bp[23]. A combination of the restriction enzymes *Hinf*I and *Rsa*I is typically used in TRF length analysis in most laboratories, including ours, for the determination of LTL. An alternative combination of *Hph*I and *Mnl*I can also be used; the difference between these enzyme mixtures is the proximity of the enzymes' terminal restriction sites to the start of the canonical region of the telomeres (**Fig. 3a**). *Hph*I/*Mnl*I cuts DNA within the noncanonical subtelomeric region, whereas *Hinf*I/*Rsa*I leaves this region intact. Thus, digestion with *Hinf*I/*Rsa*I usually results in a mean TRF length that is longer by ~1 kb than that resulting from *Hph*I and *Mnl*I digestion[24] (**Fig. 3b**). Regardless of the enzymes used to digest genomic DNA, the length of nontelomeric DNA in the TRF needs to be taken into account in estimating the absolute length of the canonical telomeric DNA.

Nontelomeric DNA is difficult to quantify and can be variable between samples, which is a limitation of the TRF assay[25,26]. The original equation for calculating the mean TRF length ($\Sigma (OD_i) / \Sigma (OD_i / L_i)$, where OD_i = optical density at position i and L_i is TRF length at position i) was based on the assumption that the subtelomeric DNA mass was small and relatively constant compared with the total mass of canonical TTAGGG repeats. Later it was realized that lengths of TTAGGG repeats on different-sized TRFs within a sample may be more similar than previously thought[25,27], leading to this equation: mean TRF length $= \Sigma (OD_i \times L_i) / \Sigma (OD_i)$[28]. Both equations are now in use, with the former likely underestimating

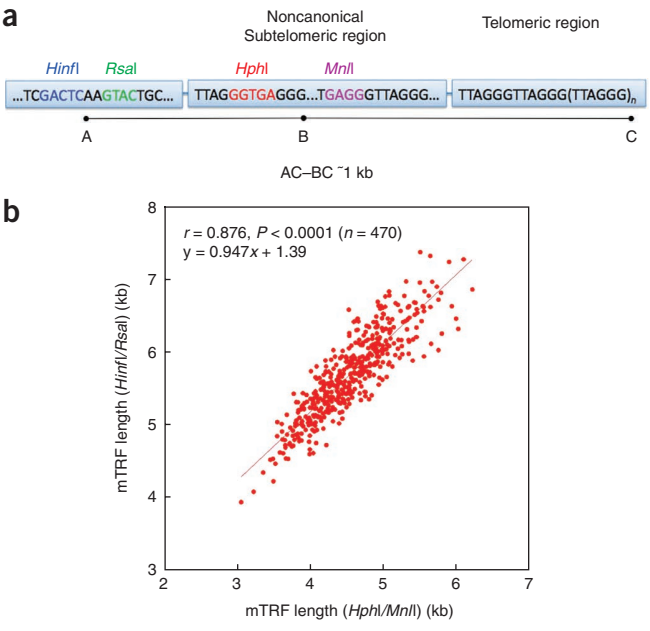

Figure 3 | Restriction digestion of genomic DNA. (**a**) Schematic representation of terminal restriction sites for restriction enzyme combinations *Hinf*I/*Rsa*I and *Hph*I/*Mnl*I. Distances AC and BC represent terminal restriction fragments (TRFs) remaining after digestion with *Hinf*I/*Rsa*I and *Hph*I/*Mnl*I, respectively. (**b**) Correlation between mean TRF length measurements obtained after digestion with alternative enzyme combinations. Measurements differ by approximately 1 kb, which corresponds to the additional stretch of genomic DNA in the noncanonical subtelomeric region that remains intact following *Hinf*I/*Rsa*I digestion.

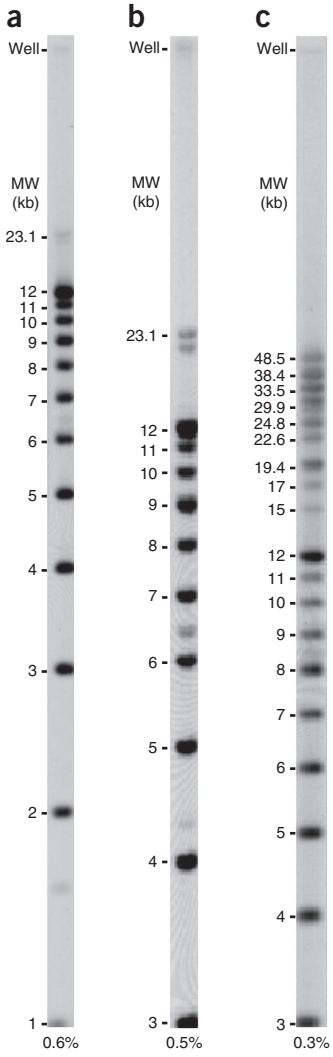

Figure 4 | Resolution of TRF length analysis in agarose gels of various percentages. (**a**) A 0.6% (wt/vol) agarose gel allows a greater resolution of shorter telomeres. It is primarily used in LTL measurements of individuals older than 80 years. (**b**) A 0.5% (wt/vol) gel, which is primarily used for studying LTL in children, young and middle-aged adults. (**c**) A 0.3% (wt/vol) gel allows the satisfactory resolution of relatively longer telomeres. This gel is appropriate for the measurement of telomere length in human sperm cells, which have longer telomeres than do somatic cells.

true telomere length and the latter overestimating it. Without experimental measurements and corrections for the length of subtelomeric DNA, the TRF method cannot give a truly accurate measurement of mean telomere DNA length.

Construction of DNA molecular weight ladder. The choice of molecular weight (MW) ladders influences the final calculation of telomeric lengths. During analysis of Southern blot films, a power function is created using the relative positions of the DNA bands in the ladder, and telomeric length is derived from this calculation. TRF length analysis for LTL is usually applied to a range of 3–20 kb; thus, the DNA ladder must include this entire range. A combination of two commercially available DNA standards is used: a 1-kb ladder that spans 0.5–12 kb and a collection of λ DNA fragments digested with *Hind*III that spans 1.25–23.1 kb.

Agarose gel electrophoresis. For standard TRF length analysis of human LTL, a 0.5% (wt/vol) agarose gel is used to resolve the digested DNA. However, the percentage of agarose gel varies in particular circumstances. When measuring very short telomeres, such as those of individuals more than 80 years of age, a 0.6% (wt/vol) gel is used; this adaptation enables measurement of telomeres as short as 1.2 kb. When measuring very long telomeres, such as those of human sperm (which can be as long as 16–17 kb (ref. 14)), we use a 0.3% (wt/vol) gel (**Fig. 4**). We have also used a 0.3% (wt/vol) gel to measure TRF length in tissues from the CAST/Ei mouse, whose telomere length is longer than that of humans but much shorter than that of the majority of other mouse strains[29]. Special care must be taken in handling low-percentage gels to ensure that they do not break during the washing and vacuum transfer steps.

Probe design and labeling. The telomere probe consists of three oligonucleotide repeats of the sequence complementary to the canonical telomeric TTAGGG sequence (i.e., CCCTAA) and is labeled at the 3′ end with digoxigenin (DIG). After the hybridization and washing steps, the DIG-labeled probe is detected with an anti–DIG-AP antibody and chemiluminescence. It is also possible to label the telomeric probe with radioactive isotopes such as ^{32}P. Although radioactive probes may enhance the sensitivity of detection, we process many thousands of samples and we avoid their use because of safety concerns. Probes for both the 1 kb and λ DNA/*Hind*III ladders are similarly labeled with DIG.

Analysis of film. Several approaches have been used to calculate final TRF length from the exposed X-ray film. All methods adjust for the higher signal intensity obtained from longer TRFs, as the telomere probe hybridizes multiple times to these fragments. One approach superimposes a premeasured grid over each lane and uses the signal intensity within each box and the corresponding MW to calculate TRF length[30]. Another approach involves including the DNA ladder within each measured sample during electrophoresis, probing and exposing the membrane twice (first with the telomere probe and then with the ladder probe), and overlaying the two films to determine the TRF length[17]. The methods described in the protocol below are those that we have found to yield the most consistent results suitable for epidemiological studies.

Internal reference. A sample of known TRF length is typically run in each Southern blot gel as an internal reference or control. It is prepared by extracting DNA from human tissue (e.g., kidney) and measuring TRF length using the methods described in the protocol below. An aliquot of this DNA is then run in the first lane of each gel and analyzed along with the other samples. This sample provides an index for the so-called 'gel effect', i.e., a drift in the results in the course of months or years of measurements. If necessary, the gel effect can be incorporated into the statistical analysis of the results.

MATERIALS

REAGENTS

- Gentra Puregene Blood Kit (Qiagen, cat. no. 158422)
- Gentra Puregene Tissue Kit (Qiagen, cat. no. 158622)
- Gentra Puregene Cell Kit (Qiagen, cat. no. 158722)
- Sodium chloride (NaCl; Fisher, cat. no. BP 358-10)
- EDTA (pH 8.0, 0.5M; Sigma, cat. no. E-7889) ! **CAUTION**: Irritant.
- Sodium dodecyl sulfate (SDS, 20% (wt/vol); Bio-Rad, cat. no. 161-0418)
- RNase (Roche, cat. no. 1119-915)
- Proteinase K (Sigma, cat. no. P6556)
- Phenol:chloroform:isoamyl alcohol (25:24:1 (vol/vol/vol); Roche, cat. no. 3117979001) ! **CAUTION** It is toxic and corrosive.
- Ethanol (Pharmco, cat. no. 111ACS200)
- Agarose (AGTC Bioproducts, cat. no. AGD1)
- Tris base (Roche, cat. no. 1814-273)
- Boric acid (Sigma, cat. no. B6768)
- Tris-borate-EDTA (10× TBE buffer; Cellgro, cat. no. 46-011-CM)
- SYBR Green I dye (Invitrogen, cat. no. S7550)
- *Hinf*I (Roche, cat. no. 1097-067)

- *Rsa*I (Roche, cat. no. 1047-671)
- Buffer A (Roche, cat. no. 1417-959)
- Ladder (1 kb; Invitrogen, cat. no. 15615-016)
- λ DNA/*Hind*III Fragments (Invitrogen, cat. no. 15612-013)
- Glycerol (Sigma, cat. no. G8773)
- Bromophenol blue (Sigma, cat. no. B8026)
- Xylene cyanol FF (Sigma, cat. no. X4126)
- Hydrochloric acid (HCl, 12 M; Fisher, cat. no. A508-212) **! CAUTION** Caustic.
- Sodium hydroxide (NaOH; Fisher, cat. no. BP359-212) **! CAUTION** Caustic.
- Tris (pH 8.0, 1 M; Fisher, cat. no. BP1758-100)
- Saline sodium citrate (20× SSC buffer; Cellgro, cat. no. 46-020-CM)
- Sarkosyl (Sigma, cat. no. L5125)
- Blocking reagent (Roche, cat. no. 1096-176)
- Maleic acid (Sigma, cat. no. M0375)
- Tween 20 (Fisher, cat. no. BP337-100)
- Anti–digoxigenin-AP antibody (Roche, cat. no. 1093-274)
- Tris-HCl (1 M; Cellgro, cat. no. 46-031-CM)
- CDP-Star (Roche, cat. no. 1759-051)
- Telomere oligonucleotide probe (MWG Operon)
- DIG Oligonucleotide 3′-End Labeling Kit (Roche, cat. no. 1362372)
- Random Primed DNA Labeling Kit (Roche, cat. no. 11004760001)
- DIG-11-dUTP (Roche, cat. no. 11093088910)
- Distilled, deionized water (ddH_2O)
- Liquid nitrogen

EQUIPMENT

- Microcentrifuge tube (0.5 ml; Fisher, cat. no. 05-408-16)
- Microcentrifuge tube (1.5 ml; Fisher, cat. no. 05-408-10)
- Spectrophotometer (NanoDrop)
- Gel electrophoresis apparatus (Scie-Plas)
- Gel combs (40 wells × 1.5 mm; Scie-Plas)
- Microwave (GE Appliances)
- Power supply (Bio-Rad)
- Gel scanner/imager (GE Healthcare Life Sciences)
- Water bath (Boekel)
- Pyrex gel box (32 cm × 23 cm × 5.1 cm; Fisher, cat. no. 15-242C)
- Gel shaker (Reliable Scientific)
- Vacuum blotter (Boekel/Appligene)
- Whatman 3MM paper (46 cm × 57 cm; Fisher, cat. no. 05-714-5)
- Serological pipettes (10 ml; Corning, cat. no. 4101)
- Nylon membrane (Roche, cat. no. 1209-272)
- UV cross-linker (UVP)
- ProBlot hybridization oven (Labnet)
- Flow-mesh membrane support (Sigma, cat. no. Z377619)
- Hybridization tubes (Fisher)
- Heat-sealable pouches (25 cm × 30 cm; Fisher, cat. no. 01-812-25BB)
- Impulse heat sealer (GHL Packaging)
- GyroMini Nutating Shaker (Labnet)
- Membrane staining box (22.5 cm × 22.5 cm × 5 cm; Sigma, cat. no. Z358304)
- Timer (Fisher)
- Autoradiography cassette (Fisher)
- X-ray film (GE Healthcare, cat. no. 28-9068-37)
- Transparency film (Corporate Express, cat. no. CEB00559)
- X-ray developer (Konica Minolta Medical)
- Densitometer (GE Healthcare)
- ImageQuant software (GE Healthcare)
- SAS software (SAS Institute Inc.)
- Typhoon 9400 (GE Healthcare)
- Mortar and pestle (for grinding frozen tissue)

REAGENT SETUP

TE buffer Mix 5 ml of 1 M Tris-HCl (pH 8.0), 1 ml of 0.5 M EDTA (pH 8.0) and 494 ml of ddH_2O; store at room temperature (RT; 20–25 °C) for up to 1 year. Alternatively, this can be purchased commercially.

5× TBE Mix 27.5 g boric acid, 54 g Tris base and 20 ml 0.5 M EDTA (pH 8.0). Adjust pH to 8.3 with NaOH and bring volume up to 1 liter with ddH_2O; store at RT for up to 1 year. Alternatively, this can be purchased commercially.

10× loading dye Mix 0.125 g bromophenol blue, 0.125 g xylene cyanol, 25 ml ddH_2O and 25 ml glycerol. Filter sterilize and store it at 4 °C for up to 6 months.

Depurination solution Add 20.8 ml of 12 M HCl to 979.2 ml ddH_2O; store at RT for up to 1 year.

Denaturation solution Add 20 g NaOH pellets and 87.6 g NaCl to 0.5 liter ddH_2O. Bring up total volume to 1 liter with ddH_2O; store at RT for up to 1 year.

Neutralization solution Mix 500 ml of 1 M Tris (pH 8.0) with 87.6 g NaCl. Add ddH_2O to a total volume of 1 liter; store at RT for up to 1 year.

20× SSC Mix 175.3 g NaCl, 88.2 g sodium citrate and 800 ml ddH_2O. Adjust pH to 7.0 with 12 M HCl and add ddH_2O to a total volume of 1 liter; store at RT for up to 1 year.

Prehybridization buffer Mix 12.5 ml of 20× SSC, 0.5 ml of 10% (wt/vol) Sarkosyl, 100 µl of 20% SDS and 37 ml ddH_2O; store at −20 °C for up to 6 months.

Wash buffer 1 Mix 100 ml of 20× SSC and 5 ml of 20% SDS with 895 ml ddH_2O; store at RT for up to 1 year.

10× Maleic acid buffer Mix 116.1 g maleic acid and 87.66 g NaCl. Add ddH_2O to a total volume of 1 liter; store at 4 °C for up to 6 months.

Blocking buffer Add 1 g blocking reagent to 100 ml fresh 1× maleic acid buffer. Heat it to 70 °C to dissolve, then cool it to RT before use; prepare fresh.

Wash buffer 2 Add 200 ml of 10× maleic acid buffer and 6 ml Tween 20 to 1,794 ml ddH_2O; store at RT for up to 1 year.

AP buffer Mix 50 ml of 1 M Tris-HCl (pH 9.5), 10 ml of 5 M NaCl and 440 ml ddH_2O; store at RT for up to 6 months.

PROCEDURE

DNA extraction ● TIMING 1–2 d

1| Total genomic DNA can be extracted from human samples by phenol-chloroform extraction (option A) or by salt precipitation (option B).

! CAUTION Informed consent must be obtained from all human subjects.

(A) Phenol-chloroform extraction

(i) Prepare fresh lysis buffer by combining 0.5 ml lysis buffer B with 2.5 µl RNase A (500 µg ml^{-1}) per sample.

(ii) Lyse human samples using methods outlined in **Box 1**.

(iii) Incubate sample tubes in a 37 °C water bath for 2 h, inverting them gently every 30 min.

(iv) Add 10 µl of 10 mg ml^{-1} proteinase K to each tube and invert 2–3 times.

(v) Incubate the lysis mixture overnight in a 50 °C water bath.

(vi) Add 500 µl phenol:chloroform:isoamyl alcohol to each tube and invert the mixture until it appears milky.
! CAUTION Phenol:chloroform:isoamyl alcohol is toxic and corrosive; this step should be performed under a chemical hood.

(vii) Spin tubes at 13,000*g* for 5 min at 4 °C.

(viii) For each sample tube, carefully remove the upper, aqueous phase into a new tube, avoiding collection of the interphase, and discard the remaining original tube.

(ix) Repeat Step 1A(vi–viii) and once again remove the fresh aqueous phase into a new tube.

(x) Precipitate DNA by adding 20 µl of 5 M NaCl and 1 ml 100% (vol/vol) ethanol per tube. Invert tubes 50–100 times or until precipitated DNA can be visualized. Spin at 13,000*g* for 5 min at 4 °C. Decant and discard the supernatant.

BOX 1 | LYSIS OF HUMAN SAMPLES

For leukocytes
1. Pellet buffy coat cells by centrifugation (e.g., 2,000*g* for 5 min) in a 1.5-ml microfuge tube.
2. Add 0.5 ml lysis buffer to the cell pellet and mix vigorously.

For cultured cells
1. Collect cells from an 80–90% confluent cell culture dish by trypsinization.
2. Pellet cells by centrifugation (e.g., 300*g* for 5 min) in a 1.5-ml microfuge tube.
3. Add 0.5 ml lysis buffer and mix vigorously.

For tissue
1. Separate frozen tissue into 50–100 µg segments (about 1 mm^3 each).
2. Place each segment in a 1.5-ml microfuge tube and freeze at −80 °C.
3. Immerse mortar and pestle in liquid nitrogen. Place frozen tissue in mortar and pulverize with pestle.
4. Add 0.5 ml lysis buffer to each tube and mix well.

(xi) Wash the DNA pellet twice by adding 1 ml of 70% (vol/vol) ethanol and centrifuging the mixture at 13,000*g* for 5 min at 4 °C. Carefully remove and discard the supernatant after each wash.
(xii) Air-dry the DNA pellet.
(xiii) Resuspend the pellet in 50–200 µl TE buffer and dissolve it by incubation in 37 °C water bath for 2 h.
■ **PAUSE POINT** DNA can be stored for up to 2 weeks at 4 °C or for longer periods at −20 °C, or preferably at −80 °C.

(B) Salt precipitation
(i) Extract DNA from cell or tissue samples using Gentra Puregene DNA extraction kits according to the manufacturer's instructions.

Determination of DNA concentration and purity ● TIMING 0.5 h

2| Determine DNA concentration using 1 µl of the extracted sample from Step 1 with a spectrophotometer by measuring absorbance (optical density, OD) at 260, 280 and 320 nm. DNA concentration (µg $µl^{-1}$) = (OD_{260} − OD_{320}) × 0.05 × DNA dilution factor. Adjust DNA concentration with TE buffer to an optimal range of 300–500 ng $µl^{-1}$.
▲ **CRITICAL STEP** A minimum of 3 µg DNA per sample is required to perform TRF length analysis.

3| Assess DNA purity by calculating the ratio of OD_{260}/OD_{280}.
▲ **CRITICAL STEP** The OD_{260}/OD_{280} ratio should be between 1.6 and 1.9 to ensure purity of the DNA preparation.

Determining DNA integrity ● TIMING 4 h

4| Prepare a 1% (wt/vol) agarose gel: combine 2.75 g agarose and 275 ml of 0.5× TBE, microwave the mixture until it dissolves and allow it to cool for 20 min. Pour it into the gel apparatus, insert a comb and allow the gel to solidify for 1 h. Remove the comb and fill the electrophoresis tank with 0.5× TBE.

5| Dilute 10 ng DNA in TE buffer to a total volume of 9 µl. Add 1 µl of 10× loading dye and mix well.

6| Load samples onto the agarose gel and run at 200–250 V for 45–60 min.

7| Dilute SYBR Green I nucleic acid gel stain 1:10,000 in 0.5× TBE. Cover the gel with staining solution and incubate at RT for 20 min with gentle agitation.

8| Image the gel by the Typhoon 9400 imaging system.
▲ **CRITICAL STEP** Sample DNA must appear as an intact, unshifted crown to be suitable for TRF length analysis (**Fig. 2**).

Genomic DNA digestion ● TIMING 16 h

9| In a 0.5-ml microfuge tube, dilute 3 µg DNA in TE buffer to a total volume of 9.1 µl. Prepare an enzyme master mix of 2 µl buffer A, 0.25 µl (10 U) *Rsa*I and 0.25 µl (10 U) *Hinf*I per sample. Add 1.3 µl master mix to each sample.

10| Digest DNA overnight in a 37 °C water bath.
■ **PAUSE POINT** Digested DNA can be stored at 4 °C for 2 d or at −20 °C.

Gel electrophoresis ● TIMING 1 d

11| Cast a 0.5% (wt/vol) agarose gel measuring 20 cm × 20 cm × 1 cm thickness (for standard LTL measurements). Combine 1.38 g agarose with 275 ml of 0.5× TBE, microwave it until dissolved, allow it to cool for 20 min and pour it into the gel apparatus. Insert a 1.5-mm × 40-well gel comb and allow the gel to solidify for 1 h.

▲ CRITICAL STEP Ensure that the gel comb is placed horizontally and does not angle downward.

12| Remove the comb and fill the electrophoresis tank with 2 liters of 0.5× TBE.

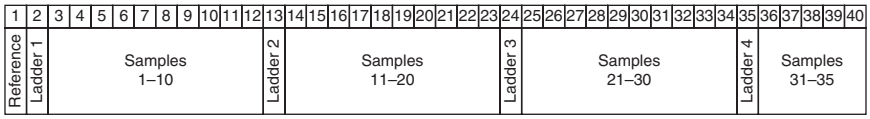

Figure 5 | Representative layout of a 40-well gel. An internal reference of known telomere length is run in the first lane, and two ladders flank each set of 10 DNA samples.

13| Prepare samples by combining 1.1 µl of 10× dye with 10 µl digested DNA.

14| Prepare MW ladder by combining 5 µl of 1-kb ladder diluted 1:10 in water, 2.5 µl of λ DNA/*Hind*III fragments diluted 1:10 in water, 4.4 µl 10× dye and 26 µl TE buffer.

15| Load 10 µl sample or ladder mixture into each well, alternating ladder and samples as illustrated in **Figure 5**.
▲ CRITICAL STEP It is essential to run the ladder in the lanes flanking every set of 10 samples so that TRF length can be accurately calculated during the analysis stage.

16| Run gel overnight (~23 h) at 2–2.25 V cm^{-1}.

Gel depurination/denaturation/neutralization ● TIMING 1.5 h

17| Place the gel in a Pyrex gel box. To facilitate DNA transfer in the next step, partially depurinate the DNA by placing the gel in 400 ml depurination solution (0.25 M HCl). Shake for 30 min, then aspirate solution with suction vacuum.

18| Denature the gel in 400 ml denaturation solution (0.5 M NaOH/1.5 M NaCl) for 30 min with gentle shaking. Aspirate the solution with vacuum.

19| Neutralize the gel in 400 ml neutralization solution (1.5 M NaCl/0.5 M Tris) for 30 min with gentle shaking. Allow the gel to remain in solution while the transfer apparatus is being prepared.

Transfer of DNA to membrane ● TIMING 2 h

20| Prepare nylon membrane (20 cm × 20 cm) and soak in 10× SSC. Prepare three sheets of Whatman 3MM chromatography paper (23 cm × 23 cm).

21| Set up vacuum transfer apparatus as detailed in **Box 2**. The final setup is illustrated in **Figure 6**.

22| Vacuum transfer DNA at 38–42 mbar for 1 h.
▲ CRITICAL STEP Check the level of transfer buffer every 15 min and ensure that the gel/membrane sandwich remains covered. Add additional 10× SSC if necessary.

23| Turn off vacuum and discard gel. Using forceps, carefully transfer the membrane onto a paper towel and allow it to dry at RT for 15 min.

24| Place the membrane, DNA side facing upward, in a UV cross-linker. Cross-link DNA onto the membrane at a setting of 120 mJ cm^{-2}. Flip the membrane (DNA side facing down) and repeat.
■ PAUSE POINT Membrane may be stored for later use. Dry membrane on Whatman paper and store at RT.

Hybridization ● TIMING 18 h

25| Place the cross-linked membrane onto a nylon mesh, carefully roll it up and place it in a hybridization tube (DNA side inward). Add 25 ml prehybridization solution. Cap the tube and rotate it for 2 h at 65 °C in a hybridization oven.

BOX 2 | VACUUM TRANSFER SETUP

1. Soak the porous 0.5-inch-thick mat in distilled water and place in the vacuum blotter.
2. Immerse the first sheet of Whatman paper in 10× SSC and place it directly in the center of the mat. Roll a 10-ml serological pipette over the mat to remove bubbles.
3. Add a second wetted sheet of Whatman paper directly over the first one and remove bubbles. Repeat with the third sheet.
4. Place presoaked nylon membrane on top of the Whatman paper, approximately 2 mm below the top edge. Roll with a serological pipette to remove air bubbles.
5. Place the thin blue rubber gasket over the membrane and mark the top left corner with a pencil. Rewet the membrane with 10× SSC. Do not allow the membrane to dry.
6. Place the gray plastic insert over the apparatus and clamp down the sides.
7. Gently slide the agarose gel over the membrane and roll with a serological pipette to remove air bubbles.
8. Cover the assembled membrane/gel sandwich with 500 ml of 10× SSC.

26| While the membrane is incubating, prepare DIG-labeled telomere probe as instructed in **Box 3**. Prepare 1 kb and λ DNA/*Hind*III ladder probes as well (**Box 4**).

27| Prepare hybridization solution by combining 25 ml fresh prehybridization buffer, 20 µl telomere probe, 3 µl of 1-kb ladder probe and 3 µl of λ DNA/*Hind*III ladder probe.

28| Discard prehybridization solution and incubate the membrane in 25 ml hybridization solution overnight at 65 °C in a rotating hybridization oven.

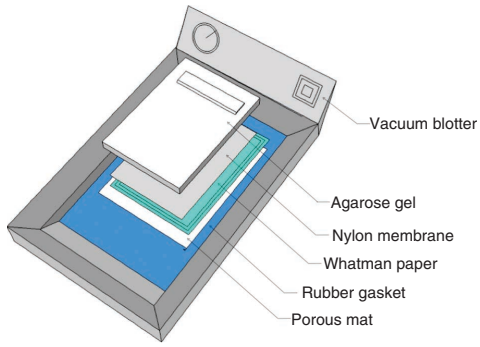

Figure 6 | Vacuum blotter setup for DNA transfer. Southern blot transfer is achieved by the action of a vacuum pump that draws the DNA downward through the agarose gel onto a positively charged nylon membrane.

Chemiluminescence detection ● TIMING 5 h

29| Transfer the membrane to a membrane-staining box. Store probe solution at −20 °C for later use. Solution may be used for two hybridization cycles before discarding.

30| Wash the membrane in 200 ml wash buffer 1 (2× SSC/0.1% (wt/vol) SDS) on a rotational shaker for 15 min. Repeat twice for a total of three washes.

31| Wash the membrane in 200 ml of 2× SSC for 15 min.

32| Prepare 100 ml fresh blocking solution (1× maleic acid buffer/1% (wt/vol) blocking reagent) and bring solution to RT.

33| Place the membrane in a heat-sealable pouch and add 60 ml blocking buffer. Remove air bubbles, seal with heat sealer and shake on a rotational shaker for 30 min at RT.

34| While the membrane is incubating, spin anti–DIG-AP antibody at 13,000*g* for 5 min at 4 °C. Add 2 µl antibody to the remaining 40 ml blocking buffer.

35| Open the heat-sealable pouch, drain the buffer and add the antibody solution. Reseal and shake the pouch for an additional 30 min.

36| Transfer the membrane to a plastic tray and wash in 200 ml wash buffer 2 (1× maleic acid buffer/0.3% (vol/vol) Tween 20) for 15 min on a shaker. Discard buffer and repeat.

37| Equilibrate the membrane in 50 ml AP buffer (0.1 M Tris/0.1 M NaCl) for 2 min. Place the membrane in a fresh heat-sealable pouch.

38| Prepare chemiluminescent substrate solution by combining 4 ml AP buffer with 40 µl CDP-Star. Pour the mixture over the membrane, remove air bubbles and heat seal the pouch.

39| Roll a 10-ml serological pipette over the sealed pouch for 5 min.

40| Prepare autoradiography cassette with two sheets of transparency film. Remove the membrane from the pouch and place between two sheets of transparency film in the autoradiography cassette. Wipe the surface of the second acetate sheet firmly with a paper towel to remove air bubbles.

BOX 3 | SYNTHESIS OF TELOMERE PROBE

1. Prepare a 1 µg µl^{-1} stock solution of telomere oligonucleotide probe with sequence $(CCCTAA)_3$.
2. Dilute the solution from the previous step 1:10 in water to prepare a stock solution of 100 ng µl^{-1}.
3. Label the telomere probe with digoxigenin using the DIG Oligonucleotide 3′-End Labeling Kit according to the manufacturer's instructions.
4. Test each batch of freshly prepared probe for labeling efficiency. Prepare serial dilutions of labeled probe (1:1–1:100,000) and spot them on membrane along with DIG-labeled control oligonucleotide. Use hybridization and chemiluminescence protocols (Steps 27–41) to expose the probe signals to film. Calculate labeling efficiency by comparing the signal intensity of the telomere probe to that of the control oligonucleotide.
5. Store labeled telomere probe in 20-µl working aliquots at −20 °C.

BOX 4 | SYNTHESIS OF LADDER PROBES

1. Combine 4 µg (4 µl) of 1-kb DNA ladder with 46 µl water in a microfuge tube on ice. In a separate tube, combine 8 µg (8 µl) of λ DNA/*Hind*III fragments with 42 µl water on ice.
2. Submerge tubes in a 100 °C water bath and boil for 10 min. Place tubes on ice.
3. Using reagents supplied in the Random Primed DNA Labeling Kit, add the following to each tube: 2 µl dATP, 2 µl dCTP, 2 µl dGTP, 1.3 µl dTTP, 0.7 µl DIG-11-dUTP and 40 µl of 2.5× random primers solution. Mix tube gently.
4. Add 2 µl Klenow enzyme (also part of the Random Primed DNA Labeling Kit) to each tube and mix gently but thoroughly. Briefly spin down tubes to collect fluid.
5. Incubate in a 37 °C water bath for 1 h.
6. Add 5 µl of 0.2 M EDTA (pH 8.0) to each tube to stop the reaction.
7. Test each probe preparation for labeling efficiency according to the manufacturer's instructions.
8. Dilute each probe 1:10 in TE buffer and store in 10-µl aliquots at −20 °C.

41| In a darkroom, place X-ray film over the blot, folding the top left corner to maintain blot orientation. Expose the membrane to the film and develop.

▲ **CRITICAL STEP** Proper exposure time should be assessed empirically until adequate visualization is achieved with minimal background.

TRF length analysis ● TIMING 3 h

42| Scan the X-ray film with a densitometer and save the digitized image.

? TROUBLESHOOTING

43| On the basis of sample characteristics (sample type, donor age, species, etc.), determine the appropriate upper and lower limits of the TRF distribution for calculation of the mean TRF length. TRF signals between 3 and 20 kb are used for LTL measurements of samples from donors younger than 80 years. However, for the measurement of LTL in samples from donors older than 80 years, the lower limit is extended to as low as 1.2 kb. In sperm TRFs, the range used is 4–48 kb.

44| Use image processing software such as ImageQuant to generate a line graph of each TRF smear by graphing OD versus DNA migration distance in pixels (**Fig. 7a**,**b**). Convert data of OD values versus DNA migration distances to OD (adjusted for background)/MW versus MW using the power function relationship between DNA migration distance (y) and MW (x) ($y = a_0 \times x^{a1}$, $r > 0.999$) (**Fig. 7c**). In general, the OD of a film in which no DNA sample is loaded is taken as a background of the entire film. In donors older than 80 years, the nadir of the range of low MW values is used as the background instead.

45| Calculate mean TRF length by applying the following equation to the TRF signal that falls within the appropriate range (as determined in Step 43):

Mean TRF length = $\Sigma (OD_i) / \Sigma(OD_i / MW_i)$, where OD is the optical density signal at position i and MW_i is the TRF length at that position.

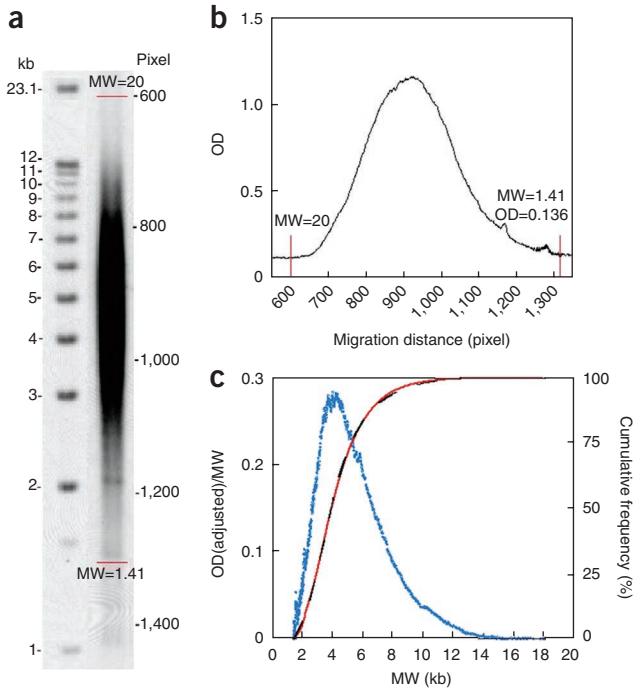

Figure 7 | Distribution of TRF lengths in a representative sample. (**a**) The solid red lines mark the scan limits used for determining the lengths of the TRFs. Note that the lower limit is extended because of the age of the donor (≥80 years). (**b**) OD versus migration distances derived from **a**. (**c**) Corresponding OD (adjusted for background) divided by molecular weight (MW; blue plot). The red line is the empirical distribution curve obtained from the scatter plot (blue plot). The dashed line is the fitted four-parameter logistic dose-response curve. The mean TRFs of the lowest 25%, the lower 50% and the median of the TRF distribution are obtained from the raw data (blue plot).

46| To estimate the distribution of TRF lengths, fix the nadir of the low MW as the background of the signal. Fit the data by the method of least squares to a four-parameter logistic dose-response distribution curve: $y = a_0 + a_1 / [1 + (x / a_2)^{a3}]$ using SAS software (**Fig. 7c**). Note that a_0 and a_1 are not constrained to 0 and 1, but the fitted parameters almost always tightly match these values. The median of the distribution is a_2 (although it can be obtained from the raw data), and a_3 is a shape parameter. The mode is derived from the maximal slope of the distribution.

● TIMING

By dividing the procedure into steps as shown in **Table 2**, the entire protocol can be carried out in 5 d (without DNA extraction) or 7 d (with DNA extraction).

TABLE 2 | TRF length analysis by Southern blot can be performed in 5–7 d.

Day	Step	Process	Time (h)
1 and 2	1	DNA extraction	24–48
	2 and 3	Determination of DNA concentration and purity	0.5
3	4–8	DNA integrity	4
	9 and 10	Genomic DNA digestion	16
4 and 5	11–16	Gel electrophoresis	24
6	17–19	Gel depurination/denaturation/neutralization	1.5
	20–24	Transfer of DNA to membrane	2
	25–28	Hybridization	18
7	29–41	Chemiluminescent detection	5
	42–46	TRF length analysis	3

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 3**.

TABLE 3 | Troubleshooting table.

Step	Problem	Possible reason	Solution
42	No signal observed	Unsuccessful transfer of DNA to membrane	Check setup of vacuum blotter apparatus
		Inefficient labeling of probe	Prepare new probe and check labeling efficiency
	Weak signal observed	Insufficient amount of DNA in sample	Check DNA concentration and repeat with 3 μg DNA
		Insufficient exposure of film	Increase exposure time until adequate visualization is achieved
	DNA appears smeared throughout lane	DNA is degraded (see **Fig. 9**)	Check DNA integrity
	Multiple bands above telomere signal	Extracted DNA contains contaminants	Check OD_{260}/OD_{280} ratio
		Inadequate digestion of genomic DNA	Digest DNA for ≥16 h. Check enzyme activity
	High membrane background	Telomere probe concentration is too high	Decrease telomere probe concentration in hybridization mixture
		Insufficient membrane washes post-hybridization	Perform an additional membrane wash in 2× SSC for 15 min
		Anti–DIG-AP antibody not centrifuged before use	Spin antibody before use

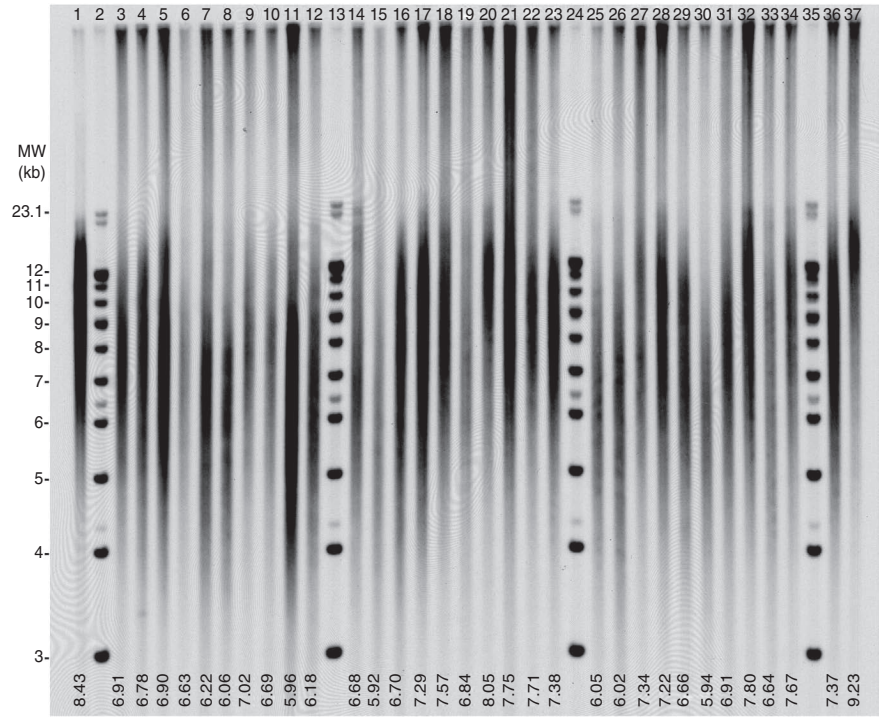

Figure 8 | Southern blot analysis of TRFs in leukocytes. A volume of 3 µg of DNA was digested with *Hinf*I/*Rsa*I and separated on a 0.5% (wt/vol) agarose gel. DNA was transferred to a membrane and probed with a telomeric probe that hybridized to TTAGGG repeats. Numbers on the left refer to molecular weight standards in kb. Lane 1 contains DNA of an internal reference sample; lanes 2, 13, 24 and 35 contain molecular weight ladders (values are indicated along the left in kb); the remaining lanes consist of DNA from 32 leukocyte samples whose leukocyte telomere lengths were determined successfully following analysis of this image. Mean TRF lengths are shown at the base of the lanes.

ANTICIPATED RESULTS

Southern blot analysis of TRF length is a technique that can measure telomere length in human cells and tissues. In epidemiological settings, LTL measurements can be used for studies across large cohorts. TRF length is calculated by integrating the optical density of each telomeric smear and interpolating the mean telomeric length (in kb) from the MW ladders. **Figure 8** shows the appearance of a typical film following successful Southern blot TRF length analysis of leukocytes. The TRF signal in each lane appears distinct with low–molecular-weight smear converting to the film background. In contrast, the signals in lanes 2 and 4 of **Figure 9** appear as diffuse smears, suggesting that the DNA was degraded before enzymatic digestion and thus rendered unsuitable for accurate TRF length determination.

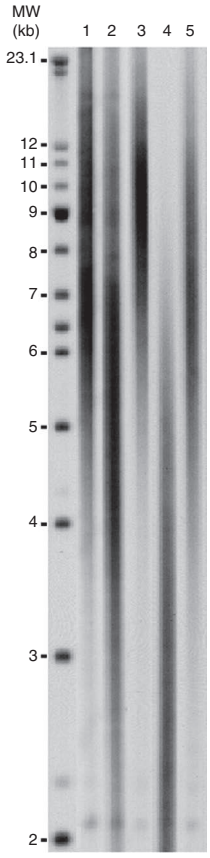

Figure 9 | Appearance of a TRF signal suggesting DNA degradation before enzymatic digestion. In contrast to the TRFs generated from intact DNA (lanes 1, 3 and 5), the TRFs of degraded DNA (lanes 2 and 4) appear as diffuse smears, the front edges of which reach the gel boundary. Note that the five samples shown in this gel correspond to those shown in **Figure 2** in the context of testing DNA integrity.

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AUTHOR CONTRIBUTIONS M.K. oversaw the development and refinement of the TRF method, generated data shown in the figures and contributed to the writing of the article. R.S. assembled the elements of the paper, focusing on the presentation and writing of the experimental design and methodology.

S.H. participated in the writing of the article, focusing on the statistical component of the introduction. J.S. oversaw the modeling and curve-fitting statistics for the TRF length distribution and contributed to the writing of the article. X.L. and X.C. routinely measured TRF length in our laboratory and provided R.S. with information to construct detailed protocols. C.H., who was a central figure in the development of the quantitative method for measuring the TRF distribution, contributed to the writing of the article. A.A. oversaw all elements of the project and participated in the writing of the article.

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